Review

A mitochondrial superoxide theory for oxidative stress diseases and aging

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Fridovich identified CuZnSOD in 1969 and manganese superoxide dismutase (MnSOD) in 1973, and proposed "the Superoxide Theory," which postulates that superoxide ($O_2^{\bullet-}$) is the origin of most reactive oxygen species (ROS) and that it undergoes a chain reaction in a cell, playing a central role in the ROS producing system. Increased oxidative stress on an organism causes damage to cells, the smallest constituent unit of an organism, which can lead to the onset of a variety of chronic diseases, such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and other neurological diseases caused by abnormalities in biological defenses or increased intracellular reactive oxygen levels. Oxidative stress also plays a role in aging. Antioxidant systems, including non-enzyme low-molecular-weight antioxidants (such as, vitamins A, C and E, polyphenols, glutathione, and coenzyme Q_{10}) and antioxidant enzymes, fight against oxidants in cells. Superoxide is considered to be a major factor in oxidant toxicity, and mitochondrial MnSOD enzymes constitute an essential defense against superoxide. Mitochondria are the major source of superoxide. The reaction of superoxide generated from mitochondria with nitric oxide is faster than SOD catalyzed reaction, and produces peroxynitrite. Thus, based on research conducted after Fridovich's seminal studies, we now propose a modified superoxide theory; i.e., superoxide is the origin of reactive oxygen and nitrogen species (RONS) and, as such, causes various redox related diseases and aging.

Key Words: **superoxide theory, MnSOD, mitochondria, ROS, oxidative stress diseases and aging**

Countless harmful substances contribute to environmental problems. These substances enter the food chain, destroy living environments, and even threaten the very survival of the human race. Contact between organisms and harmful substances can lead to diseases in organisms. Some harmful substances are linked to reactive oxygen generators and ultimately cause cellular damage. However, organisms possess biological defenses against these processes. Harmful substances and biological defenses battle inside cells, and these interactions can result in organisms maintaining their "normal" status. This review focuses on biological defenses, especially antioxidant enzyme systems.

Studies have reported that reactive oxygen causes aging and a number of diseases; for example, rheumatism, hepatitis, enteritis, and carcinogenesis.[1] It is now known that many neurological diseases are caused by reactive oxygen species (ROS); for example, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS) and the like, which are caused by abnormalities in biological defenses or increased intracellular reactive oxygen levels. Increased oxidative stress on an organism causes damage to cells, the smallest constituent unit of an organism, which can lead to the onset of a variety of diseases.

The definition of a free radical and a list of reactive oxygen species are included in Table 1.[2] Due to the emergent role of nitric oxide ($NO^{\bullet}$) in oxidative stress related diseases, reactive nitrogen cascades are sometimes included in reactive oxygen

Table 1. Reactive oxygen species

Free radicals: a free radical is any species capable of independent existence that contains one or more unpaired electrons. (B. Halliwell and J. M. C. Gutteridge, *Free Radicals in Biology and Medicine*, 2007)

Reactive Oxygen Species	
$O_2^{\bullet-}$	superoxide
$HO^{\bullet}$	hydroxyl radical
$^1O_2$	singlet oxygen
H_2O_2	hydrogen peroxide
$ONOO^-$	peroxynitrite

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Table 2. Antioxidant defense systems

Enzymes
• Mn Superoxide Dismutase (MnSOD) - Mitochondria
• CuZnSOD - Cytosol
• Glutathione Peroxidase - Cytosol and Mitochondria
• Peroxiredoxins - Cytosol, Extracellular Space and Mitochondria
Non-enzyme
• Vitamins A, C, E
• Glutathione
• Polyphenols
• Coenzyme Q_{10}

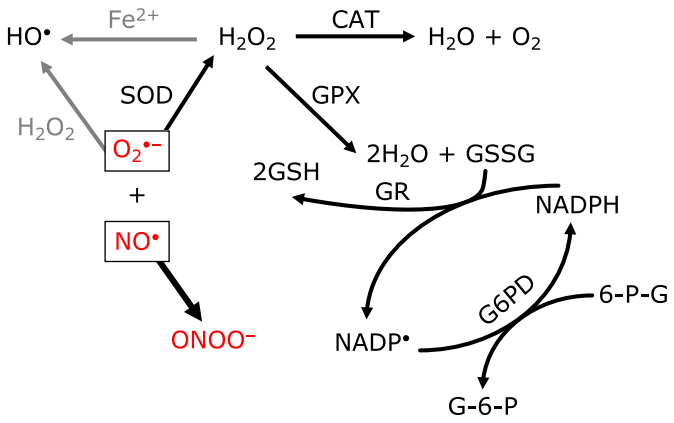

Fig. 1. Intracellular antioxidant enzymes and their chain reactions. Superoxide ($O_2^{\bullet-}$), predominantly induced from the mitochondrial electron transport chain (ETC), reacts with nitric oxide ($NO^{\bullet}$) and forms peroxynitrite ($ONOO^-$). Peroxynitrite, a potent oxidant, then induces apoptosis or necrosis. MnSOD, which locates in mitochondria, eliminates $O_2^{\bullet-}$ and inhibits binding with $NO^{\bullet}$.

cascades. Against those oxidant reactions, biological defense systems exist in cells, including enzyme-based systems and non-enzyme-based systems, as shown in Table 2. The relationship between intracellular antioxidant systems and the functions of these systems are shown in Fig. 1.

A New Mitochondrial Superoxide Theory

In 1969, when Fridovich discovered superoxide dismutase (CuZnSOD), an enzyme that eliminates superoxide,[3] he established that superoxide ($O_2^{\bullet-}$) is an important substance that is responsible for initiating a series of ROS. The history of this discovery is shown in Table 3. Fridovich also discovered manganese superoxide dismutase (MnSOD), in 1973.[4] However, a research published approximately 10 years later showed that superoxide has low activity and poor reactivity, and concluded that the superoxide theory was invalid.[5] Rate constants for the principal radicals with ascorbic acid in ion-balanced solutions, shown in Table 4,[6] confirm that superoxide has low activity and poor reactivity. It was believed that this was also the case in bio-environments. In the same period, studies showing that nitric oxide, which plays an important role in the physiological activities of organisms, may participate in superoxide-mediated reactivity and received much attention.[7] It was eventually established that superoxide and nitric oxide readily react to form an extremely reactive substance called peroxynitrite ($ONOO^-$) (Fig. 2).[8] In 1997, we demonstrated that cells transfected with MnSOD genes

Table 3. History of superoxide theory of oxygen toxicity

- Discovery of Superoxide dismutase (**CuZnSOD**); McCord JM, Fridovich I. *J Biol Chem* 1969; **244**: 6049–6055.
- Discovery of Mitochondrial Superoxide dismutase (**MnSOD**); Weisiger RA, Fridovich I. *J Biol Chem* 1973; **248**: 4793–4796.
- Superoxide ($O_2^{\bullet-}$) is not a strong oxidant; Sawyer DT, Valentine JS. *Acc Chem Res* 1981; **14**: 393–400.
- Endothelium-derived relaxing factor (EDRF) is nitric oxide (**NO**); Palmer RMJ, Ferrige AG, Moncada S. *Nature* 1987; **327**: 524–526.
- Peroxynitrite (**ONOO**$^-$) is a potent oxidant and nitrating agent; Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA, *Proc Natl Acad U S A* 1990; **87**: 1620–1624.
- **MnSOD** protects against cell death: **Superoxide** induction from **mitochondria** relates apoptosis; Majima HJ, Oberley TD, Furukawa K, Mattson MP, Yen H-C, Szweda LI, St. Clair D. *J Biol Chem* 1998; **273**: 8217–8224.
- Nobel prize laureates: Murat F, Ignarro L, Furchgott R. 1998.

Table 4. Second-order rate constants for the reaction of the free radicals with ascorbate

Free Radicals	k_{obs} ($M^{-1}s^{-1}$)
$HO^{\bullet}$	1.1×10^{10}
$RO^{\bullet}$ (*tert*-butoxy radical)	1.6×10^{9}
$ROO^{\bullet}$ (alkylperoxyl radical)	$1–2 \times 10^{6}$
$GS^{\bullet}$ (glutathionyl radical)	6×10^{8}
$O_2^{\bullet-}$	1×10^{5}

exhibited resistance to apoptotic death caused by alkaline, which is an oxidative stress condition.[9] MnSOD precursor protein has a mitochondrial targeting signal (MTS) composed of 24 amino acids that transport MnSOD protein from cytosol into the matrix of mitochondria (Fig. 3). MTS is cleaved, and then mature MnSOD protein eliminates superoxide inside the matrix of mitochondria, protecting mitochondrial DNA by inhibiting superoxide from reacting with nitric oxide (Fig. 4). These results suggest that superoxide generated from mitochondria controls subsequent oxidative reactions. As a result of this finding, we propose "A Mitochondrial Superoxide Theory."[10]

The Role of Superoxide Dismutase Enzymes

Enzymes scavenging superoxide are referred to as SODs (EC 1. 15. 1. 1). Three types of SODs have been identified in mammals: CuZnSOD (SOD1), MnSOD (SOD2) and ECSOD (SOD3, extracellular SOD).[3,4,11,12] The oxidation-reduction active centers of these SODs are zinc and copper for CuZnSOD and ECSOD, and manganese for MnSOD. CuZnSOD is homodimeric and present in cytoplasm. The genes of CuZnSOD are present in chromosome 21 in humans and in chromosome 16 in mice. CuZnSOD is localized in cytosol, but recent studies have shown that it is also localized inside mitochondria, in intermembrane space,[13–16] where superoxide anions are released from Complex III. SOD1 is linked to amyotrophic lateral sclerosis (ALS),[17–20] aging[21] and Alzheimer's disease.[22] The enzyme MnSOD is located in mitochondria, and it is homotetrameric. Genes of this enzyme are present in chromosome 6 in humans and in chromosome 8 in mice. The enzyme has a mitochondrial localizing signal, comprised of 24 amino acids that target the protein to mitochondria. Upon reaching its destination, the signal part is cleaved and the mature protein becomes localized inside the mitochondria.[9,10]

ECSOD is homotetrameric and a glycosylated CuZnSOD. Genes of ECSOD are present in chromosome 4 in humans and in chromosome 5 in mice. ECSOD is found predominantly in the

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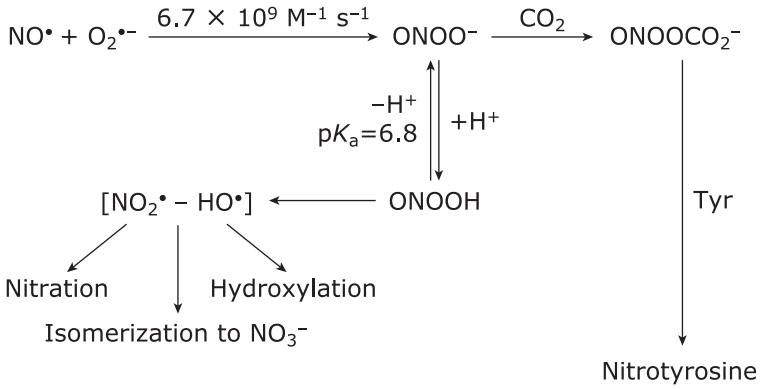

Fig. 2. Formation of peroxynitrite ($ONOO^-$) and its further reactions. Nitrates and/or hydroxylates of proteins, lipids and DNAs, by formation of $NO_2^•$ and $HO^•$ (adapted from Beckman, *et al.*[8]).

MLSRAVCGTSRQLAPALGYLGSRQ
ATGTTGAGCCGGGCAGTGTGCGGCACCAGCAGGCAGCTG
GCTCCGGCTTTGGGGTATCTGGGCTCCAGGCAGAAGCACA
GCCTCCCCGACCTGCCCTACGACTACGGCGCCCTGGAACC
TCACATCAACGCGCAGATCATGCAGCTGCACCACAGCAA
GCACCACGCGGCCTACGTGAACAACCTGAACGTCAACGA
GGAGAAGTACCAGGAGGCGTTGGCCAAGGGAGATGTTAC
AGCCCAGATAGCTCTTCAGCCTGCACTGAAGTTCAATGGT
GGTGGTCATATCAATCATAGCATTTTCTGGACAAACCTCA
GCCCTAACGGTGGTGGAGAACCCAAAGGGGAGTTGCTGG
AAGCCATCAAACGTGACTTTGGTTCCTTTGACAAGTTTAA
GGAGAAGCTGACGGCTGCATCTGTTGGTGTCCAAGGCTCA
GGTTGGGGTTGGCTTGGTTTCAATAAGGAACGGGGACACT
TACAAATTGCTGCTTGTCCAAATCAGGATCCACTGCAAGG
AACAACAGGCCTTATTCCACTGCTGGGGATTGATGTGTGG
GAGCACGCTTACTACCTTCAGTATAAAAATGTCAGGCCTG
ATTATCTAAAAGCTATTTGGAATGTAATCAACTGGGAGAA
TGTAACTGAAAGATACATGGCTTGCAAAAAG

Fig. 3. MnSOD has a mitochondria targeting signal (MTS). The sequence consists of an alternating pattern (amphipathic helix) composed of 24 amino acids, which includes three positively charged peptides (arginine [R]).

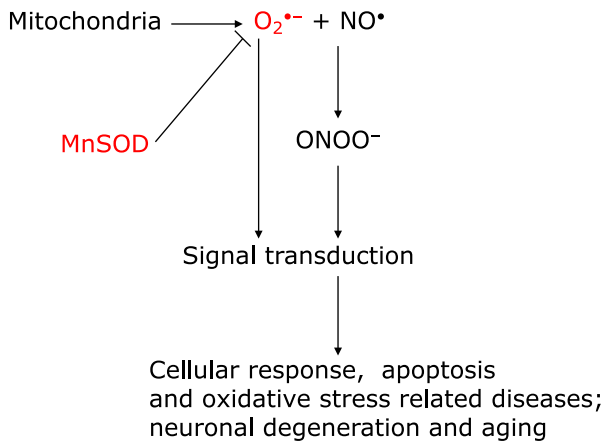

Fig. 4. A Mitochondrial Superoxide Theory. The binding reaction of superoxide ($O_2^{•-}$) and nitric oxide ($NO^•$) forms peroxynitrite ($ONOO^-$) (amendment to Motoori, *et al.*[71]).

extracellular matrix of tissues and situated to prevent cell and tissue damage initiated by extracellularly produced ROS. ECSOD may protect against pulmonary fibrosis and other extracellular superoxide mediated diseases.[12,23,24]

Mitochondrial dysfunction has been linked to aging and a wide range of degenerative and metabolic diseases, including cancer.[25] In cells, superoxide is produced from oxygen molecules by xanthine oxidase, NADPH oxidase and mitochondrial electron transfer systems. Superoxide produced in mitochondria is generated by electrons leaking from the electron transfer system, which is located in the inner membrane of mitochondria. These electrons are then captured by molecular oxygen and become superoxide.[26–29] It is estimated that an adult at rest utilizes 3.5 ml O_2/kg/minute or 352.8 L/day (assuming 70 kg body mass) or 14.7 mol/day.[30] If 1% makes superoxide, a human produces 0.147 mol/day or 53.66 moles/year or about 1.72 kg/year of superoxide. During physical exertion, this would increase up to 10-fold, assuming that the 1% still applied. Therefore, superoxide generated from mitochondria in normal cells is present in large quantities in cells. SODs eliminate these superoxides. The importance of these SODs can be seen from the results of experiments using genetic knockout mice.[31,32] Cutler reported on the correlation between SOD activity and survival time using 2 rodents and

12 primate species of maximum lifespan potentials (MLSPs) ranging from 3.5 to 95 years.[33] Recently, Page *et al.*[34] reported no correlation between antioxidant enzyme activities and longevity among 14 mammalian and avian species with MLSPs ranging from 3 years to over 100 years. However, they also found that MnSOD and catalase positively correlated with MLSP only for brain tissue. Although the Mitochondrial Free Radical Theory of Aging (MFRTA) remains unproven due to various complex data,[35] it seems that MnSOD could play an important role in aging. In other words, a control of ROS generated from mitochondria could be an important factor for the aging process. These reports, then, suggest that superoxide has an important effect in cells, which in turn implies that "the superoxide theory" is still valid. The regulation of reactive oxygen has implications for many types of degenarative conditions, including aging.

Mitochondria-localized Manganese Superoxide Dismutase (MnSOD)

While CuZnSOD is present in the cytoplasm and forms a dimer, MnSOD forms a tetramer (homotetrameric), as described previously. The gene encoding MnSOD is located on chromosome 6 in humans and chromosome 8 in mice.[36] The cDNA of the MnSOD gene consists of 666 bps, the first 72 bps of which correspond to 24 amino acids MTS for translocation of the precursor protein into the mitochondrion (Fig. 3).[37] MTS is followed by the start signal codon (AUG; methionine). This mitochondrial translocation signal contains many basic amino acids and thus is a cation as a whole. Because the mitochondrial outer membrane is negatively charged, the speculation was that it electrically attracts MTS. Subsequent studies identified TOM and TIM in the mitochondrial outer and inner membranes, which were found to attract proteins into the mitochondrion.[38–40] To attract proteins into the mitochondrion, these molecules have a stable primary structure via the binding of a chaperone protein to the amino acid chain.[38–40] An MnSOD molecule attracted into the mitochondrial matrix is cleaved at the MTS portion and assumes a three-dimensional structure, which then forms a tetramer with manganese at the active center to form the mature and active form of the molecule.[38–40]

Several studies conducted in the 1970s demonstrated a leakage of electrons from some proteins present in the mitochondrial inner membrane, particularly complexes I and III, and the resulting production of superoxide.[28,29,41] However, the exact percentage of all intracellularly generated active oxygen species accounted for by mitochondria-derived superoxide remains unclear. The intracellular systems that produce superoxide include peroxidases (non-specific),[42] xanthine oxidase,[43,44] nitric oxide synthase (NOS),[45–50] aldehyde oxidase,[51,52] NADPH oxidase,[53] fumarate reductase,[54] heme proteins[55] and the mitochondrial electron transport system.[56] We have demonstrated that the mitochondrion is the most abundant source of superoxide of all these systems.[56] Generation of active oxygen ($O_2^{\bullet-}$) from the mitochondrial electron transport system causes oxidative stress to the cell and subsequently induces oxidative-related diseases.[26,57,58] Although other antioxiant enzymes play important roles in mitochondria, e.g., peroxiredoxin (Prx)[59,60] and glutathione peroxidase (GPx),[61,62] as mentioned above, MnSOD, an enzyme localized in the mitochondrion, and scavenging superoxide, has several important roles.[4] The following findings of MnSOD suggest the critical role of MnSOD in the survival of aerobic life: (1) Escherichia coli and yeasts lacking the MnSOD gene are highly sensitive to oxidative stress.[63–65] (2) MnSOD gene knockout mice can only survive 10–18 days after birth, with pathological findings of cardiomyopathy, fatty liver, skeletal muscle acidosis and degeneration of neurons in the central nervous system due to mitochondrial disorder, suggesting a critical role of the enzyme.[31,32] Superoxide can directly oxidize [4Fe–4S] of aconitase, etc., to form hydrogen peroxide (H_2O_2) with subsequent release of Fe^{2+}. The cluster is also in complex I, and so aconitase and complex I are inactivated in SOD2 KO mice, which is important for superoxide toxicity.[66,67] (3) Cells transfected with MnSOD cDNAs are resistant to paraquat,[68] tumor necrosis factor,[69,70] doxorubicin,[69] mitomycin C,[69] irradiation,[69,71–76] alkaline treatment,[9] hypoxic condition,[77] ischemic reperfusion,[78] smoking toxicity[79] and radiation carcinogenesis.[80] (4) Human MnSOD gene transgenic mice show reduced severity of hyperbaric oxygen-induced pulmonary damage[81] and adriamycin-induced myocardial damage.[82] Free radicals generated from mitochondria could play roles in any kind of cell death; i.e., apoptosis, necrosis and autophagy.[83,84]

How SOD Works to Remove Oxidative Stress in Mitochondria

Several hypotheses describe the important role MnSOD plays as an antioxidant.[76] Hydroxyl radicals are produced from the Fenton reaction (1) or Haber-Weiss reaction. Thus, they are formed H_2O_2 and superoxide ($O_2^{\bullet-}$).

The Fenton reaction is defined as:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + HO^{-} \quad \text{..... (1)}$$

The Haber-Weiss reaction is initiated by

$$Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2 \quad \text{..... (2)}$$

The overall reaction is:

$$O_2^{\bullet-} + H_2O_2 \rightarrow HO^{\bullet} + HO^{-} + O_2 \quad \text{..... (3)}$$

Cells with more MnSOD should generate more H_2O_2 due to more substrate being available for reactions, and more $HO^{\bullet}$ could be produced by the Fenton and Harber-Weiss reactions. However, the absence of superoxide prevents the first step of the Haber-Weiss reaction and thus $HO^{\bullet}$ formation is reduced. This is consistent with the general observation that the level of ROS ($HO^{\bullet}$), subsequent lipid peroxidation and apoptosis are decreased by MnSOD overexpression. Thus, the Fenton reaction alone does not correspond with these results and does not explain the observed reduced amounts of ROS (mostly $HO^{\bullet}$) by MnSOD transfection. The excess production of H_2O_2 by MnSOD could be quickly detoxified by GPx by reducing it to water.[85] This reaction could be accompanied by glutathione, of which the level for most cells is ~5 mM, an excess amount for the reaction.[85]

Superoxide radicals can react with $NO^{\bullet}$ to form $ONOO^{-}$ with a diffusion-controlled rate, because $NO^{\bullet}$ has an unpaired electron.[86] $ONOO^{-}$ is a potent biological oxidant that has recently been implicated in diverse forms of free radical-induced tissue injury.[8,87] The reaction of $ONOO^{-}$ with membrane lipids induces a phospholipid membrane peroxidation product even without iron being present.[88] Various aldehydes are generated as final products when lipid hydroperoxides break down. Among them, HNE is a highly toxic nine-carbon α,β-unsaturated aldehyde that can be generated by the peroxidation of ω-6 unsaturated fatty acids, such as arachidonic acid and linoleic acid.[89,90] Peroxynitrous acid (protonated forms of $ONOO^{-}$:ONOOH) subsequently produces $HO^{\bullet}$ and nitrogen dioxide radicals ($NO_2^{\bullet}$), resulting in oxidation and nitration, respectively.[8] Thus, MnSOD inhibits the formation of $ONOO^{-}$ by decreasing superoxide levels that prevent the peroxidant effects produced by $ONOO^{-}$ (Fig. 2 and 4).

Free radicals generated from mitochondria could play a role in many kinds of cell death; i.e., apoptosis, necrosis, autophagy.[83,84] In our published paper that reported the relationship between ROS generated from mitochondria and apoptosis,[9] we showed an electron microscopic picture of mitophagy impacted by an alkaline condition, whereas MnSOD transfected cells seemed normal, as

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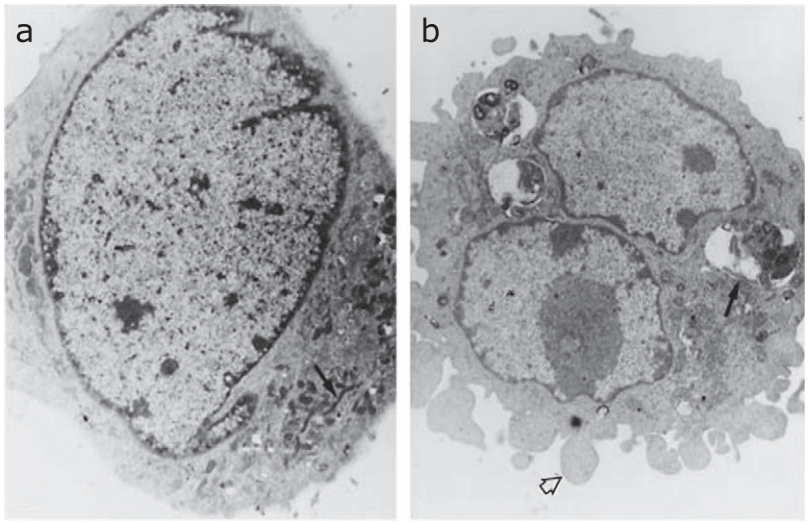

Fig. 5. Ultrastructural analysis of cells cultured at pH 8.3 for 6 h. Electron microscopy of (a) SOD transfected cells that appear normal with normal mitochondria (arrow) and (b) NEO cells show accumulation of lysosomes with membrane debris observed internally (arrow). The cell surface demonstrates prominent membrane blebs (arrowhead). Mitochondria show focal swelling and loss of cristae. Focal condensation of chromosomal material is present. This research was originally published in J Biol Chem., Majima HJ, Oberley TD, Furukawa K, Mattson MP, Yen H-C, Szweda LI, St Clair DK: Prevention of mitochondrial injury by manganese superoxide dismutase reveals a primary mechanism for alkaline-induced cell death. *J Biol Chem* 1998; **273**: 8217–8224. Reprint from ref. 9 with permission.

shown in Fig. 5. These findings indicate that MnSOD is essential for maintenance of life and cellular resistance to oxidative stress in the presence of oxygen, and suggest that superoxide generated from mitochondria plays an important role in oxidative stress and its related diseases, including aging.

Conclusion

Mitochondrial ETC generates superoxide under physiological conditions, and oxidative stress increases ROS production. Mitochondria are the major source of intracellular superoxide production, and damage of mtDNA appears to damage mitochondrial DNA encoded proteins in ETC, causing more superoxide to be produced. Reducing excess amounts of mitochondria-generated superoxide seems important to protecting against oxidative stress related diseases. The reactivity of superoxide is relatively low, as shown in Table 4. However, when elevated levels of $NO^{\bullet}$ are present, nitric oxide binds to mitochondrial ETC-generated superoxide. Subsequently, $ONOO^{-}$ is formed with the rate constant close to that of the reaction between hydroxyl radical ($HO^{\bullet}$) and ascorbic acid (Table 4, Fig. 2). Then, $ONOO^{-}$ produces hydroxyl radicals and nitrogen dioxide, and oxidizes and nitrates DNAs, lipids and proteins, etc., and induces apoptosis, autophagy, mitophagy and necrosis (Fig. 2 and 5). MnSOD exists in mitochondria (Fig. 3) to block the binding of mitochondrial ETC-generated superoxide with nitric oxide (Fig. 4). This theory, called "A Mitochondrial Superoxide Theory" (Fig. 4), could explain the initiation of numerous chronic diseases, such as aging and carcinogenesis.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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